will remain important for specialized applications requiring femtomole sensitivity.

See also: **III/Amino Acids:** Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Amino Acids and Derivatives: Chiral Separations. Amino Acids and Peptides: Capillary Electrophoresis.**

Further Reading

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Hušek P and Macek K (1975) Gas chromatography of amino acids. *Journal of Chromatography* 113: 139.

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Liquid Chromatography

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The first approach to the automatic liquid chromatography (LC) of amino acids (AAs) – known today as ion exchange chromatography (IEC) – was published by Spackman *et al.* in 1958. In over 40 years later, it now takes less than 5 min (**Figure 1**) to separate and quantitate the essential protein AAs instead of 2 days. Early separations were carried out by post-column derivatization.

Over the last 20 years LC has offered unlimited possibilities in both the preparative and analytical scale. The wide choice and sophisticated columns, detectors, derivatization procedures, development of modern instrumentation and data-handling systems reduce time and costs, and give versatility and automation in Good Laboratory Practice (GLP)- controlled conditions for selectivity, sensitivity and reproducibility. It is the responsibility of the researcher to choose the most appropriate method for the given task. The most popular LC method for analysis of both free AAs (present in many natural matrices, biological fluids and tissues, feed and foodstuffs) and of those constituents of protein hydrolysates is now reversed-phase (RP) chromatography after pre-column derivatization of the AAs.

Numerous methods for derivatization are available in the literature. This article will discuss the advantages and drawbacks of the commonly used derivatives.

Current trends in AA analysis identify the best conditions for enantiomer separation and the development of LC-mass spectrometry (LC-MS).

LC of Underivatized AAs

To attain one of the main advantages of LC – separating the 'classical 20' as underivatized AAs – has appealed to chromatographers. In spite of a number of efforts, the simultaneous LC of underivatized AAs has remained of secondary importance. Determination of a few selected AAs, such as tryptophan or sulfur-containing AAs, has proved to be fruitful for special tasks.

The aim of various investigations was to render unnecessary the time-consuming derivatization techniques. However, the characteristics of the free AAs are considerably different from each other and their various structural properties did not permit their easy resolution. Thus, in attempting to achieve better separation of free AAs, further means of discrimination were needed. For this purpose special techniques have been introduced, such as the use of various phase systems, ion pair and ligand exchange chromatography, column-switching techniques or anion exchange chromatography with electrochemical detection.

The solvent-generated ion exchange phase system ensured the gradient elution of 19 AAs (**Figure 2**A): some, but not all, are baseline-separated. A simple isocratic method using aqueous, copper acetate/alkylsulfonate additives containing acetate buffer (pH 5.6) as mobile phase, a conventional RP column and UV detection (230–240 nm) at different temperatures and varying the concentrations of additives was unable to separate the classical 20 protein amino acids. Significant improvement in the separation can be obtained by column switching (Figure 2B), as well as by using an anion exchange column, a quaternary

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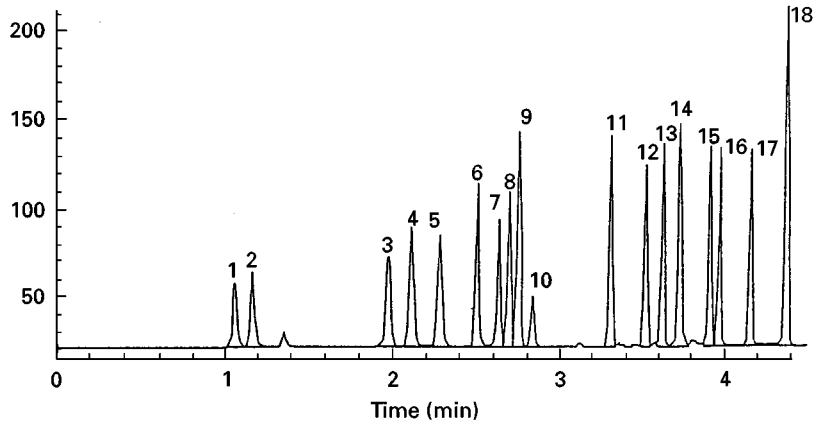

Figure 1 Separation of the phenylthiocarbamyl AAs separated on TSKgel Super-ODS (for details see Table 3). Peaks: 1, ASP; 2, Glu; 3, Ser; 4, Gly; 5, His; 6, Arg; 7, Thr; 8, Ala; 9, Pro; 10, NH_4^+; 11, Tyr; 12, Val; 13, Met; 14, Cys; 15, Ile, 16, Leu; 17, Phe; 18, Lys. (Reproduced with permission from Tosohaas, The Bioseparation Specialist, (1995) Catalogue p. 157, Figure 9/2.)

gradient mobile phase and pulsed amperometric detection (Figure 2C). For tryptophan and the sulfur-containing AAs (cysteine/cystine, methionine, glutathione, etc.), the fast isocratic elution of the underivatized samples has gained wide acceptance and is a powerful tool in their quantitation.

Tryptophan can be measured directly, within 8 min, in neutralized alkaline hydrolysates of feed and foodstuffs, using an RP column, 5% methanol containing acetate buffer (pH $\sim$ 4.0) and UV detection at 280 nm. The pulsed amperometric detection of sulfur-containing AAs, at the low pmol level, was carried out with an Au working- and an Ag/AgCl reference electrode, subsequent to their separation on both cation exchange and on RP columns, applying as mobile phase 0.1 mol L^{-1} $HClO_4$/0.15 mol L^{-1} $NaClO_4$/5% ACN.

LC of Derivatized AAs

Derivatization studies have concerned the optimization of parameters, such as the yield and stability of derivatives, to separate and quantitate all AAs with a simple and fast elution procedure.

Post-column Derivatization (Table 1, Figure 3)

Post-column derivatization was the first development of IEC in the area of RP/high performance liquid chromatography (HPLC), in its pioneer period. It took time to develop pre-column derivatization concepts which resulted in considerable advantages.

Drawbacks of the post-column techniques (**Table 1**) are long elution times and the need for costly devices, such as a delivery system for the derivatizing reagent (one or more extra pumps); (a) mixing chamber for the column effluent and the reagent(s); special thermostable reactors (packed bed, air-segmented and/or coil reactors) ensuring the necessary delay for quantitative reactions accompanied with as small band broadening as possible. Last but not least, the mobile phase was probably incompatible with the derivatizing reagent. The preferred mobile phase might be inappropriate for the optimum conditions of derivatization reaction. The early and current stages of post-column methods can be illustrated by elution followed by post-column reaction with ninhydrin (NHYD; **Figure 3**A), with *o*-phthaldialdehyde/β-mercaptoethanol (OPA/MCE; Figure 3B), or with 1,2-naphthoqunione-4-sulfonyl chloride (NQS; **Figure 3**C). All three types of derivatives have been separated in most cases on ion exchange resin columns from the early 1970s. Recent post-column methods are without exception, slow separations (**Table 1**). However, the efficiency of the recently published methods of NHYD derivatives using short columns is superior, determining 59 compounds in 150 min and 40 compounds in 120 min respectively.

Pre-column Derivatization (Tables 2 and 3; Figures 4–6)

Pre-column derivatization offers numerous advantages. It requires less equipment and allows the evaluation of the derivatives in an easier way from the point of view of their selectivity, sensitivity, various means of detection, derivatization yield, stability and storability. All of these phenomena can be controlled and improved by use of modern instrumental techniques and computerization, both individually and simultaneously. Potential disadvantages in pre-column derivatization as procedures can be completely avoided: contamination from the reagents (due to their insufficient purity) and loss of analyte

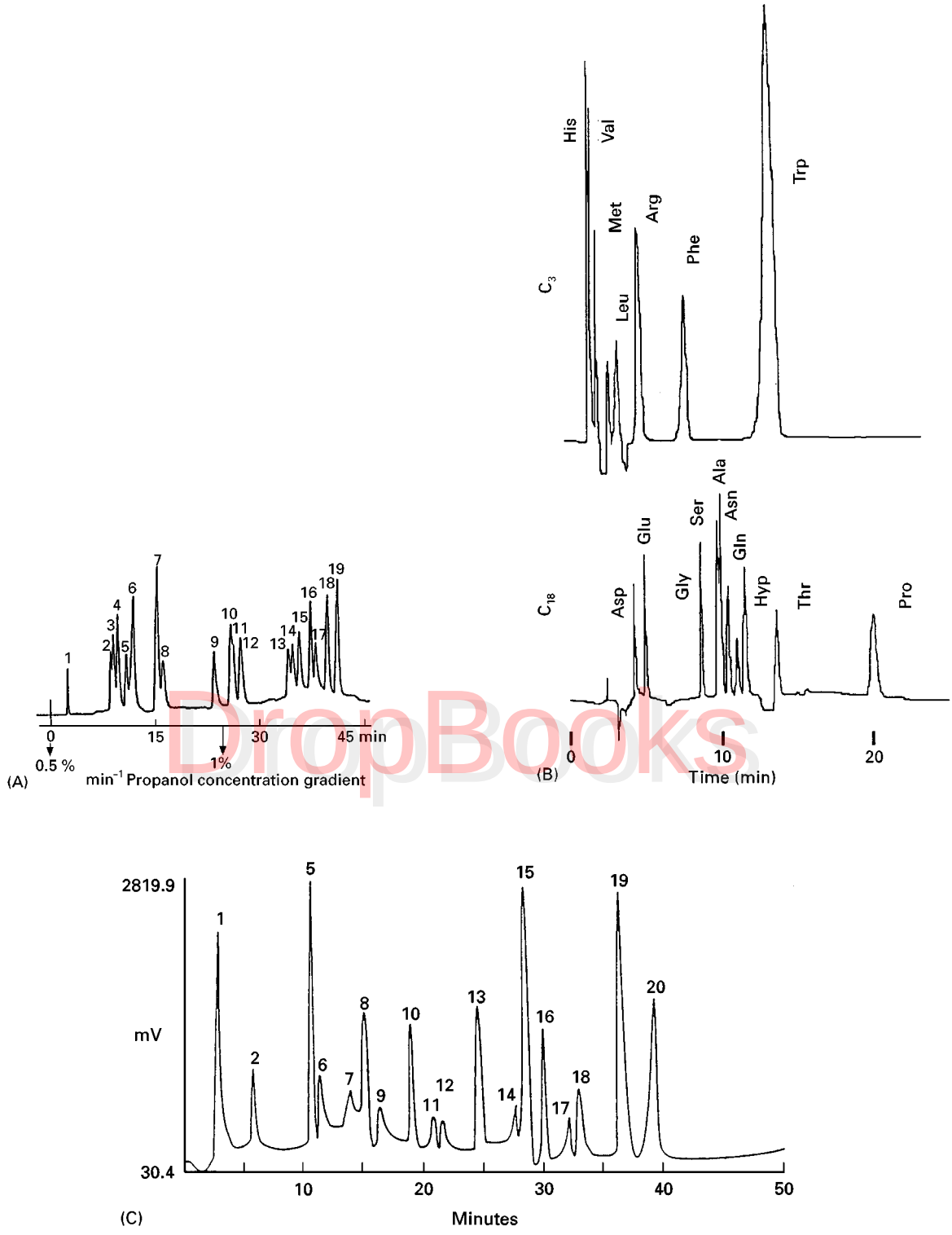

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Figure 2 LC of underivatized AAs. (A) Separation of a test mixture using *n*-propanol gradient. Column: 250 × 3 mm, RP-8; temperature = 25°C. Peaks: 1, $CySO_3H$; 2, Asp; 3, Ser; 4, Glu; 5, Thr; 6, Gly + Pro; 7, Ala; 8, Cys; 9, NH_4^+; 10, Tyr; 11, Val; 12, Met; 13, Ile; 14, Phe; 15, Leu; 16, His, 17, Lys; 18, Trp; 19, Arg. (Reproduced with permission from Kraak JC *et al.* (1977) *Journal of Chromatography* 142: 671.) (B) Chromatogram of standard AAs using a column-switching technique. First column, Inertsil C_3; second column, Inertsil ODS-2. (Reproduced with permission from Hanai T and Hirukawa M (1988) *Journal of Liquid Chromatography* 11: 1741.) (C) Chromatogram of AAs obtained by pulsed amperometric detection. Peaks : 1, Arg; 2, Lys; 3, Gln; 4, Asn; 5, Thr; 6, Ala; 7, Gly; 8, Ser; 9, Val; 10, Pro; 11, Ile; 12, Leu; 13, Met; 14, system peak; 15, His; 16, Phe; 17, Glu; 18, Asp; 19, Cys; 20, Tyr. (Reproduced with permission from Frankenberger WT Jr and Martens DA (1992) *Journal of Liquid Chromatography* 15; 423.)

Table 1 Advances in the LC of post-column derivatized AAs, obtained with *o*-phthaldialdehyde/β-mercaptoethanol (OPA/MCE), with ninhydrin (NHYD) or with 1,2-naphthoquinone-4-sulfonate (NQS)

Author and date	*Column size*			*Type*	*Eluents (elution temperature °C)*	*Detector UV (nm) FEx/Em*	*Reagent (°C)*	*Analyte (nmol L^{-1})*	*RSD %*	*Matrix*	*No. of AAs/ elution time (min)*
	cm	*mm*	*μm*								
Moore, 1958	150	0.9	40	Amberlite IR-120, IE	Citrate buffers, 0.2 mol L^{-1}: pH 3.25 for first day (30°C), and pH 4.25 for the second day (55°C)	UV 440, 570	NHYD (–)	100–3000	—	AAs in hydrolysates	20/ 24–48 h
Grunau, 1992	15	3	5	Pickering 'fast run'	Pickering Eluents A (Li280), B (Li750), C (RG003) (42°C)	UV 570	NHYD (130°C)	20	—	Plasma AAs	59/ ~150
Iwase, 1995	6	4.6	3	2622, Hitachi, IE	Five eluents: PF-1–PF-4, PF-RG, cont. Li salts, ethanol, benzyl alcohol, thiodiglycol, Brij-35 buffer with pH 2.8, 3.7, 3.6, 4.1, -; (gradient programme: 28–40°C)	UV 440,570	NHYD (130°C)	50	< 3	Plasma AAs	40/120
Roth, 1973	25	6		Aminex 6, IE	Citrate buffers: pH 3.20, 4.25 and 6.40 for 40, 60 and 70 min (34°C for 100 min, then raised to 55°C)	F —	OPA/MCE (55°C)	10	—	Model study	14/170
Elrifi, 1986	60	9	—	IE	Pierce Pico-Buffer system, Li citrate buffers; pH of A,B,C,D and E = 2.9, 3.1, 3.5, 3.4 and 2.3; temperature gradient: 0–44 min (34°C), 44–128 min (63°C)	F* No data	OPA/MCE (40°C)	0.63–45.0	—	AAs in foods	23/128
Haginaka, 1988	30 +guard 3	4.6 4.6	5 5	ODS-5	A: 15 mmol L^{-1} Na octane sulfonate/ 21 mmol L^{-1} H_3PO_4/9 mmol L^{-1} NaH_2PO_4/CH_3OH (20/20/20/1, v/v), pH 2.8; B: as A, except (1/1/1/6, v/v), pH 4.2 (60°C)	F 340/450	OPA/MCE (60°C)	0.25–2.5	< 4.5	AAs in hydrolysates	18/ ~120
Møller, 1993	15 +guard 2	3 3	5 5	Pickering, IE	A: 0.24 mol L^{-1} Li citrate, pH 2.27, B: 0.64 mol L^{-1} Li citrate pH = 7.50 (50°C)	F 340/448	OPA/MCE (4°C)	0.6	< 11	Physio-logical AAs	39/180
Saurina, 1994	15	4.6	5	Spherisorb ODS 2	A: 20 mmol L^{-1} H_3PO_4 + 20 mmol L^{-1} NaH_2PO_4 + 15 mmol L^{-1} SDS; B: 25 mmol L^{-1} H_3PO_4 + 25 mmol L^{-1} NaH_2PO_4 + 18.5 mmol L^{-1} SDS/PrOH (4:1, v/v); (50°C)	UV 305	NQS (65°C)	32	< 5	AAs in food + feed[a]	18/105

No data available; IE, ion exchange resin; [a]hydrolysates.

from incomplete interaction, undesirable side reactions and sample handling losses.

Although numerous pre-column derivatization techniques have been introduced in the last 30 years, none complies with the criteria of an ideal procedure: providing rapid and quantitative interaction in aqueous media, permitting mild conditions, ensuring interaction with both primary and secondary AAs and resulting in single and stable derivatives in the case of all AAs.

OPA Derivatives (Table 2 and Figure 4)

The pioneering work of Roth (1971) on the very fast reaction of AAs in aqueous solutions with ophthalaldehyde mercaptoethanol (OPA/MCE), detectable by both UV and fluorescence, without the need to remove excess reagent, represented a great advance. Because of the different stability of the isoindoles obtained from the reaction of AAs with OPA/MCE, pre-column derivatization with 3-mercaptopropionic acid (MPA) and several *N*-alkyl-L/D-cysteines was proposed. The OPA/MPA and OPA/*N*-acetyl-L-cysteine (NAC) reagents provide more stable isoindoles compared to those formed with OPA/MCE, and the optical resolution of enantiomeric amino acids with OPA/NAC, as well as with other *N*-alkyl-L/D-cysteine reagents, has opened a new area in enantiomer separation of AAs. Due to robotic autosamplers which provide excellent reproducibility for even moderately quantitative interactions, most AA analyses are

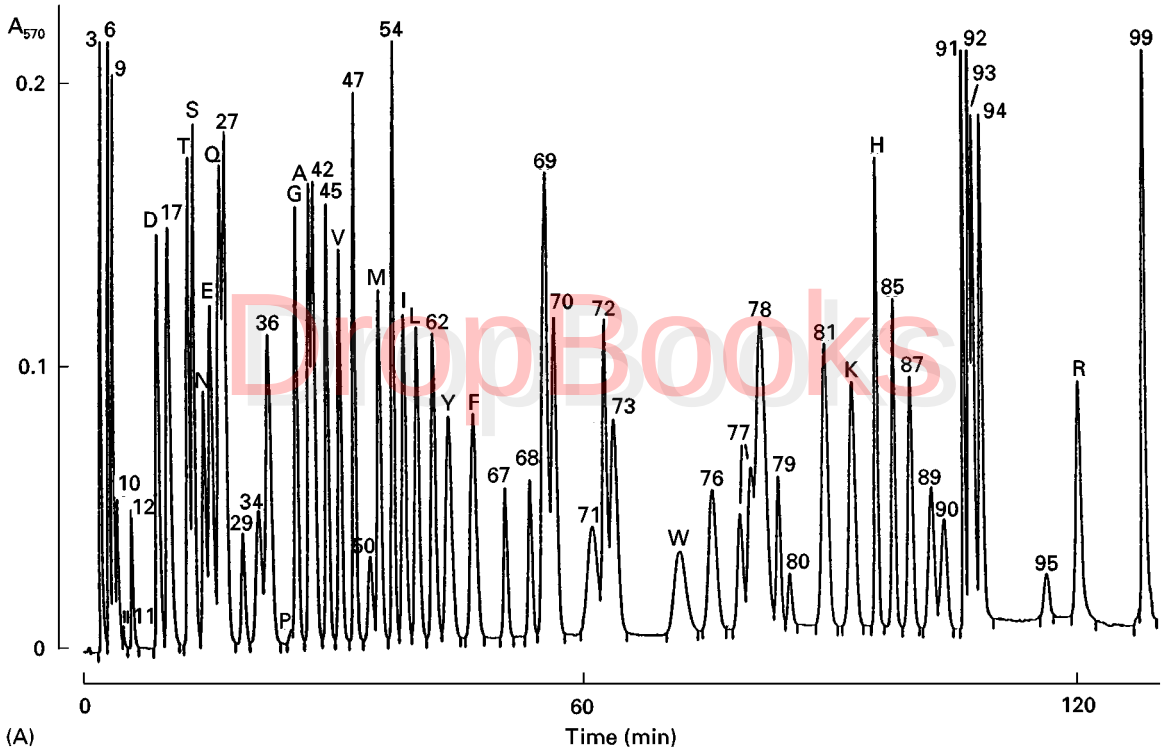

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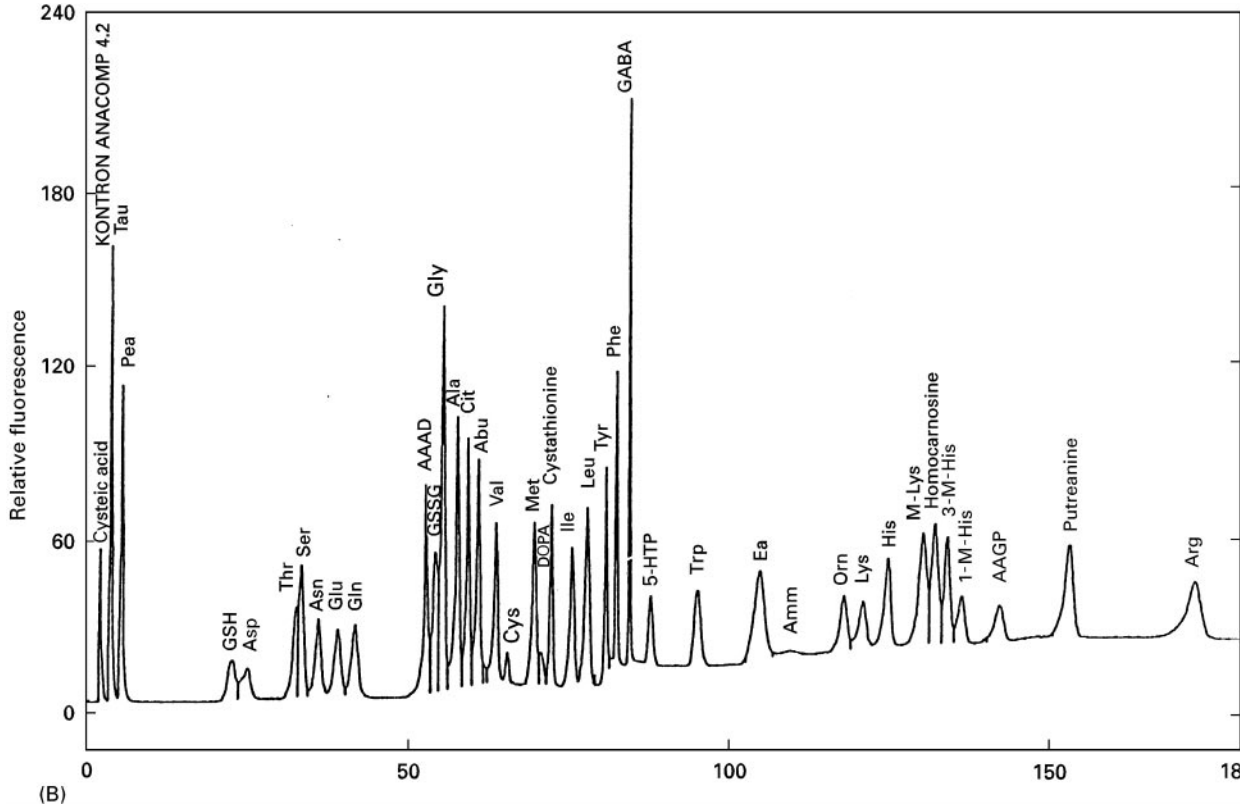
Figure 3 LC of post-column derivatized AAs (for details see Table 2). (A) Chromatographic profile of 59 AAs and related compounds. Peaks: 3, *o*-phospho-DL-serine; 6, taurine; 9, *o*-phosphoethanolamine; 10, N^{α}-(1-D-mannityl)-L-glutamine (mannopine); 11, urea; 12, β-ciano-L-alanine; D, L-aspartic acid; 17, *o*-acetyl-L-serine; T, L-threonine; S, L-serine; N, L-asparagine; E, L-glutamic acid; Q, L-glutamine; 27, L-homoserine; 29, sarcosine; 34, DL-α-aminoadipic acid; 36, *S*-methyl-L-cysteine; P, L-proline; G, glycine; A, L-alanine; 42, L-citrulline; 45, L-α-aminobutyric acid; V, L-valine; 47, L-cystine; 50, α-methyl-DL-methionine; M, L-methionine; 54, L-cystathionine; I, L-isoleucine; L, L-leucine; 62, L-norleucine; Y, L-tyrosine; F, L = phenylalanine; 67, β-alanine; 68; DL-aminoisobutyric acid; 69, DL-homocystine; 70, δ-aminolevulinic acid; 71, 5-hydroxy-L-tryptophan; 72, γ-aminobutyric acid; 73, DL-kynurenine; W, L-tryptophan; 76, ethanolamine; 77, δ-hydroxylysines (DL- and DL-allo); 78, ammonia; 79, ε-amino-*n*-caproic acid; 80, creatinine; 81, L-ornithine; K, L-lysine; H, L-histidine; 85, 3-methyl-L-histidine; 87, 1-methyl-L-histidine; 89, L-carnosine; 90, L-anserine; 91, L-canavanine; 92, *S*-methyl-DL-methionine; 93, L-α-amino-β-guanidinopropionic acid; 94, L-leucinamide; 95, N^{G1}-dimethyl-L-arginine; R, L-arginine; 99, L-homoarginine. (Reproduced with permission from Grunau JA and Swiader JM (1992) *Journal of Chromatography* 594: 165.) (B) Separation of OPA/MCE derivatives by gradient IEC chromatography. (Reproduced with permission from Møller SE (1993) *Journal of Chromatography* 613: 223.) (C) Determination of AAs by ion pair liquid chromatography with post-column derivatization using 1,2-naphtoquinone-4-sulfonate (NQS). Peaks: 1, Asp; 2, Ser; 3, Glu; 4, Gly; 5, Thr; 6, Ala; 7, Pro; 8, Tyr; 9, Met; 10, Ile; 11, Phe; 12, Leu; 13, Nle; 14, Trp; 15, His; 16, Orn; 17, Lys; 18, Arg. Line = elution gradient profile. (Reproduced with permission from Saurina J and Hernández-Cassou (1994) *Journal of Chromatography* 676: 311.)



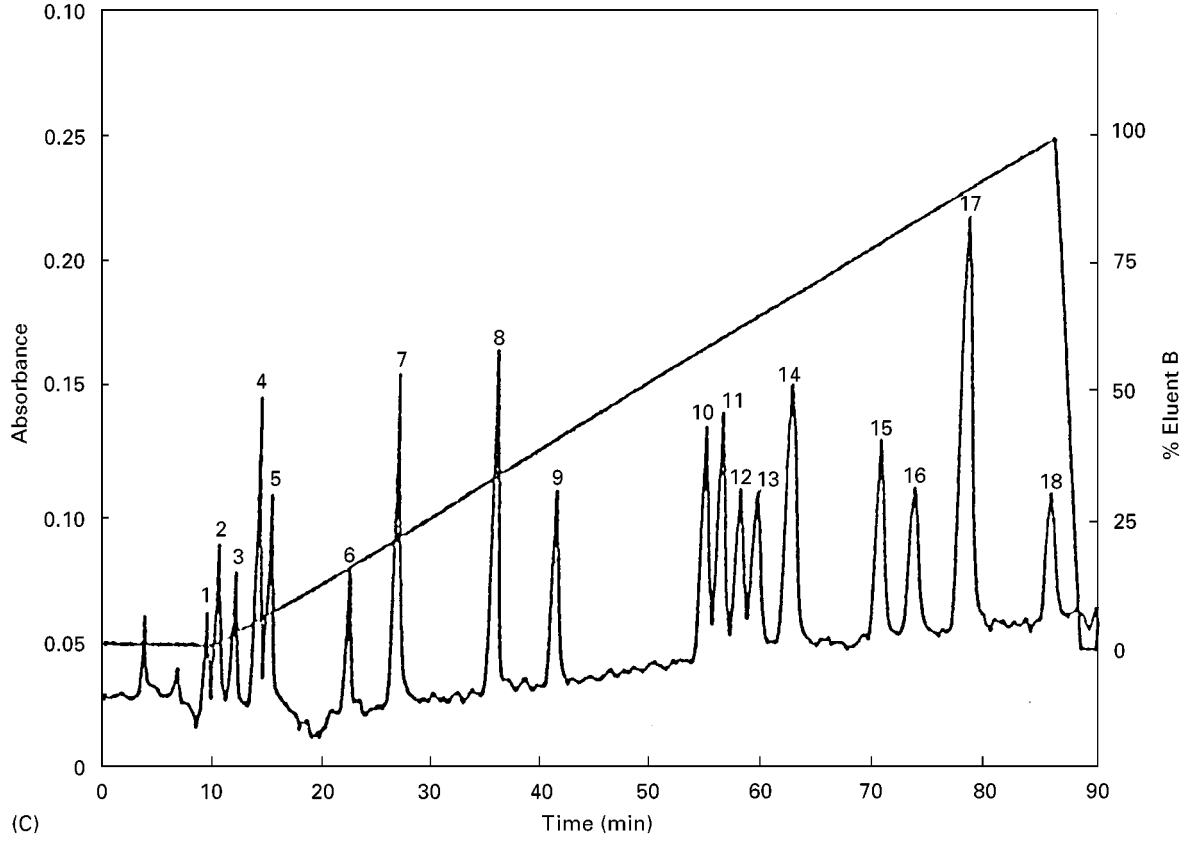


Figure 3 *Continued*

Table 2 Advances in the LC of pre-column derivatized AAs, obtained with OPA/MCE, OPA/3-ethanethiol (OPA/ET), OPA/mercaptopropionic acid (OPA/MPA), OPA/*N*-acetyl-L-cysteine (OPA/NAC), with OPA/isobutyryl-L/D-cysteine (OPA/NIBC) or with OPA/MPA/fluorenylmethylchloroformate (OPA/MPA/FMOC)

Author and date	*Column size*			*Type*	*Eluents (elution temperature °C)*	*Detector UV (nm) FEx/Em*	*Reagent (°C)*	*Analyte (pmol L^{-1})*	*RSD %*	*Matrix*	*No. of AAs/elution time (min)*
	cm ×	*mm,*	*μm*								
Jones, 1983	75 +guard 45	4.6 2.1	3 40	Ultrasphere ODS	A: THF/CH_3 OH/NaAc (pH 7.2) = (5:95:900, v/v) B: CH_3 OH; (−)	F 305–395 420–650	OPA/MCE (−)	0.1–80	<1.5	AAs in hydrolysates	48/50
Fekkes, 1995	12.5	4.6	5	Spherisorb ODS-2	A: (pH 6.72–6.77 and B: (pH 5.95–6.00): 250 mmol L^{-1} Na_2HPO_4/ 250 mmol L^{-1} propionic acid/ACN/THF/H_2O = (20:20:7:2:51, v/v) C: ACN/CH_3OH/DMSO/ H_2O = (28:24:5:43, v/v); (25–35°C)	F 337/452	OPA/MCE (3°C)	50	<2	Plasma AAs	40/49
Hill, 1979	30	3.9	—	μ-Bondapak C-18	A: 12.5 mmol L^{-L} Na_2HPO_4 (pH 7.2) B: A eluent/ACN in gradient; (−)	F 229/470	OPA/ET (−)	5	—	AAs in human serum	20/40
Eslami, 1987	50	4.5	3	ODS IBM	Buffer: ~2 mol L^{-1} Na_2 HPO_4 (pH 7) A: ACN/H_2O/buffer = (50:425:25, v/v) B: ACN/H_2O/buffer = (275:200:25, v/v); (22°C)	F 330/480	OPA/ET (−)	40–100	—	Model study	22/14
Godel, 1984	25	4	4	Supersphere CH-8	A: 12.5 mmol L^{-1} Na_2HPO_4 (pH 7.2) B: 12.5 mmol L^{-1} Na_2HPO_4 (pH 7.2)/ ACN-(1:1, v/v); (−)	F 330/445	OPA/MPA (−)	1–10	<4.2	AAs in biological fluids	28/40
van Eijk, 1993	15 +guard 1	4.6 4	2–3	Spherisorb ODS-2	A: 12.5 mmol L^{-1} Na_2HPO_4 (pH 7.0) + 7 mL THF/1 l eluent B: 12.5 mmol L^{-1} Na_2HPO_4 (pH 7.0)/ ACN/THF = (57:43:7, v/v); (35°C)	F 335/440	OPA/MPA (−)	35	<3	Plasma AAs	30/28
Teerlink, 1994	10 + guard 1	4.6 2	3	Microsphere ODS	A: 4.5 mmol L^{-1} K_2HPO_4 (pH 6.9) + 2 mL THF/1L B: 4.5 mmol L^{-1} K_2HPO_4 (pH 6.9)/CH_3OH/ACN = (50:35:15, v/v); (−)	F 230/389	OPA/MPA (−)	100	<3.2	Plasma AAs	25/17
Schuster, 1989	20 20	2.1 4.6	5 5	Hypersil ODS	Protein hydrolysates, A: 30 mmol L^{-1} NaAc cont. 0.5% THF (pH 7.2); ACN/0.1 mol L^{-1} NaAc = (4:1, v/v); (42°C) Plasma AAs, A: 60 mmol L^{-1} NaAc cont. 0.6% THF (pH 8.0); B: ACN/0.1 mol L^{-1} NaAc/ CH_3OH = (14:4:1, v/v); (43°C)	UV 338/266 F 230/455 266/310	OPA/MPA/FMOC (4°C)	UV: 2–5 F: 0.02–0.05	<2.5	AAs in protein hydrolysates Plasma AAs	19/20 38/60

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Table 2 *Continued*

Author and date	*Column size*			*Type*	*Eluents (elution temperature °C)*	*Detector UV (nm) FEx/Em*	*Reagent (°C)*	*Analyte (pmol L^{-1})*	*RSD %*	*Matrix*	*No. of AAs/elution time (min)*
	cm ×	*mm,*	*μm*								
Bartók, 1994	10	4	3	Hypersil ODS	A: 18 mmol L^{-1} NaAc (pH 7.2) + 0.02%(v/v) TEA + 0.3% THF (v/v) B: ACN/CH_3OH/NaAc 0.1 mol L^{-1} (pH 7.2) = (2:2:1, v/v); (40°C)	F 340/450 264/313	OPA/ MPA/ FMOC (4°C)	50	<1.1	Plant AAs	21/8

Indications as in Table 1.

performed with OPA derivatives. The essential shortage of an OPA/SH-group reagent (reactive toward the primary AAs only) was eliminated by Shuster's principle – the automatic two-step pre-column derivatization method applying the OPA/MPA/fluorenylmethyl chloroformate (FMOC) reagent, which also ensures derivatization of the secondary AAs. A high speed elution of OPA/MPA/FMOC derivatives was shown recently (**Table 2**, **Figure 4**: 19 compounds/8 min). Evaluating the improvements between the corresponding early and recent procedures, in the newer methods shorter, thermostated columns of smaller particle size with autosamplers are now used, giving greater sensitivity and reproducibility.

Phenylthiocarbamyl (PTC), FMOC, 1-*N,N′*-Dimethylaminonapthalene-5-sulfonyl (DANS) and Dimethylaminoazobenzenesulfonyl (DABS) Derivatives (Table 3, Figures 1, 5 and 6)

Judging by the number of publications in the last decade, the interest in the PTC and FMOC derivatives has proved to be lasting, while the application of DANS and DABS derivatives is decreasing. However, in the direct enantiomer separation of AAs, the use of DANS derivatives is preferred.

The reaction of AAs with *phenylisothiocyanate* (**Table 3**, **Figures 1** and **5**), in water-free media at ambient temperature is quantitative and fast (10 min), resulting in the highly stable single PTC derivatives (except for cyst(e)ines in hydrolysates which elute in one to four peaks). The excess reagent is removed by vacuum, and the PTC derivatives can be stored in the freezer for an unlimited time, and for a day after dissolution in buffer at 4°C. UV detection at 254 nm allows their quantitation in the low pmol range. The short PicoTag and the short TSK gel columns can separate 17 AAs within 12 min and 4.5 min, respectively.

The first LC separation of the strongly fluorescent DANS AAs has been used earlier in protein chemistry and in thin-layer chromatography. The decreased popularity of this technique in LC can be

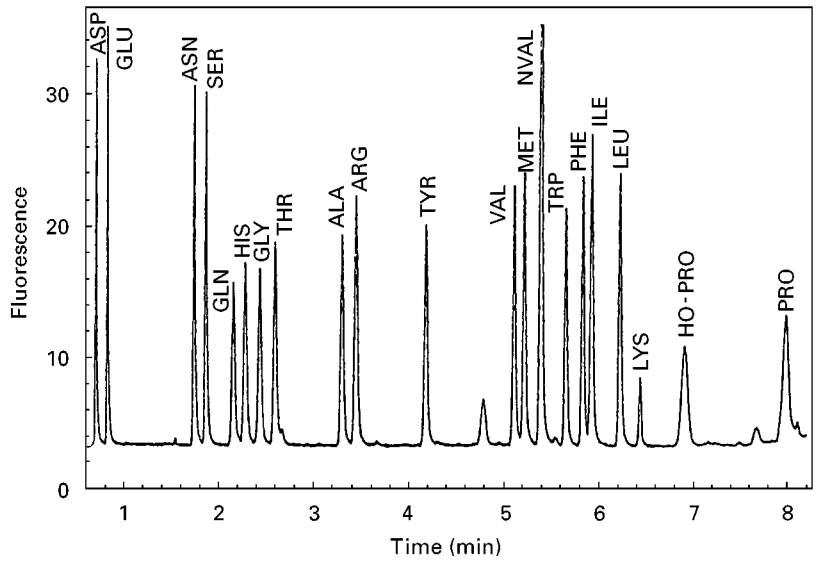

Figure 4 High speed RP-HPLC analysis of the OPA/MPA/9-fluorenylmethyl chloroformate derivatives. (Reproduced with permission from Bartók T *et al.* (1994) *Journal of Liquid Chromatography* 17: 4391.)

Table 3 Advances in the LC of pre-column derivatized AAs, obtained with phenylisothiocyanate (PITC), 5-dimethylaminonaphtalene-1-sulfony1-Cl (DANS), 4-dimethylaminoazobenzene-4-sulfonyl-Cl (DABS) or with 9-fluorenylmethyl chloroformate (FMOC)

Author and date	*Column size*			*Type*	*Eluents (elution temperature °C)*	*Detector UV (nm), FEx/Em*	*Reagent (°C)*	*Analyte (pmol L^{-1})*	*RSD %*	*Matrix*	*No. of AAs/elution time (min)*
	cm × mm,		*μm*								
Koop, 1982	25	4.6	5	Ultrasphere ODS	A: 70 mmol L^{-1} NaH_2 PO_4 (adjusted to pH 6.45 with TEA) B: ACN; (27°C)	UV 254	PITC (−)	6000	—	AAs in protein hydrolysates	18/130
Tosohaas, 1995	10	4.6	2	TSKgel Super-ODs	A: 50 mmol L^{-1} NaAc (pH 6.0)/ACN = (97:3, v/v) B: 50 mmol L^{-1} NaAc (pH 6.0)/ACN = (40:60, v/v); (40°C)	UV 254	PITC (−)	250	—	Model study	17/4.5
Shang, 1996	15	3.9	5	PicoTag ODS	A: NaAc (pH 6.4) B: ACN; A and B performed in gradient (38°C)	UV 254	PITC (−)	5	< 1.9	AAs in kelp	17/12
Bayer, 1976	50	3	10	LiChrosorb, RP 8	Eluent 10 $mmol^{-1}$ Na_2HPO_4/CH_3OH = (50:20, v/v) to which 1.5 mL CH_3OH/min is added (45°C)	F 340/510	DANS (amb)	0.1	—	Model study	17/40
Martins, 1996	15	3.9	4	Nova Pak C 18	A: 30 mmol L^{-1} phosphate buffer (pH 7.4) + 5 mL CH_3OH + 6.5 mL THF adjusted to 100 mL with distilled water B: CH_3OH/H_2O = (70/30, v/v); (25°C)	F 338/445	DANS (40°C)	60	—	AAs in polypetides	17/35
Chang, 1983	—	—	5	—	A: 25 mmol L^{-1} NaAc (pH 6.5) containing 4% dimethylformamide B: ACN (40°C)	UV 436	DABS (70°C)	5	—	AAs in protein hydrolysates	17/40
Yang, 1993	15	4.6	5	Hypersil ODS	A: 25 mmol L^{-1} NaAc (pH 6.35) containing 4% dimethylformamide B: ACN (40°C)	UV 436, 580	DABS (70°C)	50	—	AAs in polypetides	17/40
Einarsson, 1983	50 500	4.6 2.26	3 5	Spherisorb ODS-2	Eluent: 20 mmol L^{-1} NaAC buffer (pH 4.08–4.31)/ACN gradient; (−)	F 265/315	FMOC (−)	(−)	< 6.6	AAs in protein hydrolysates, in urine	17/10 and 33/100
Qu, 1996	15	4.6	5	Hypersil ODS	A: 30 mmol L^{-1} phosphate buffer (pH 6.5) in 15% CH_3OH (v/v) B: 15% CH_3OH (v/v) C: 90% ACN (v/v); (38°C)	F 270/316	FMOC (−)	125	< 1.0	AAs in protein hydrolysates, biological samples	15/35
Bank, 1996	15	4.6	5	Micropak ODS-80TM	A: 20 mmol L^{-1} citric acid/ NaAc buffer (pH 2.85); B: 20 mmol L^{-1} NaAc (pH 4.5)/CH_3OH = (80: 20, v/v); A, and B both, cont. 0.01% (w/v) NaN_3 + 5 mmol L^{-1} $(CH_3)_4$ NCI; C: ACN; (40°C)	F 254/630	FMOC (−)	50	< 3.6	AAs in protein hydrolysates	21/35

Indications as in Table 1.

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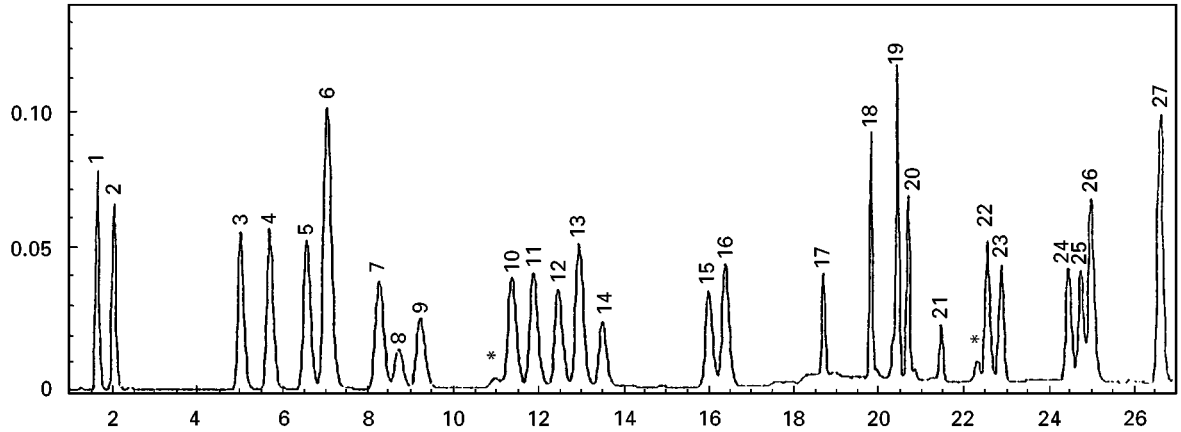

Figure 5 Separation of 27 phenylthiocarbamyl AAs. Column 150 + (20 guard) × 4 mm, C_{18} Hypersil 5 μm, temperature, 50°C, eluent A: 0.05 mol L^{-1}. NaAc pH 7.2; B: A eluent/acetonitrile/methanol = 46/44/10 (pH = 7.2), flow rate: 2.1 mL min^{-1}. Peaks: 1, aspartic, 2, glutamic acids; 3, hydroxyproline; 4, serine; 5, glycine; 6, asparagine; 7, β-alanine; 8, glutamine; 9, homoserine; 10, γ-aminobutyric acid (GABA); 11, histidine; 12, threonine; 13, alanine; 14, 1-amino-1-cyclopropane carboxylic acid (ACPCA); 15, arginine; 16, proline; 17, homoarginine; 18, tyrosine; 19, valine; 20, methionine; 21, cyst(e)ine; 22, isoleucine; 23, *n*-leucine; 24, phenylalanine; 25, tryptophan; 26, ornithine; 27, lysine. *system peaks. (Reproduced with permission from Vasanits A and Molnár-Perl (1998) *Journal of Choromatography* 832:109.)

explained by its two main disadvantages: long reaction times, or elevated temperatures for derivatization, and generation of fluorescent side products (DANS hydroxide, DANS amide) and interference from excess reagent. The disturbing effect of these compounds cannot be completely eliminated and they elute between the AA derivatives. No significant improvement has been obtained and cannot be expected.

DABS AAs were first separated applying precolumn labelling. Derivatization was performed in $Na_2CO_3/NaHCO_3$ buffer, at pH ~ 8.9 with DABS chloride dissolved in acetone under continuous stirring at 80°C for 10 min. DABS AAs can be stored for 4 weeks in solution at 25°C, without any changes. In spite of the unique stability of DABS AAs in aqueous media, and the improvement in their chromatographic conditions, the use of DABS AAs is dwindling.

FMOC was introduced in 1983, as a fluorescent labelling agent, reacting rapidly with both primary and secondary AAs, under mild conditions (borate buffer, pH 7.7–8.0) to give stable derivatives. The excess reagent is extracted by pentane. Recent derivatization studies have shown that, depending on the time (2 and 40 min) and pH (8.0 and 11.4), considerable differences can be found. At pH ~ 8, acidic AAs manifest low responses, and slow reaction

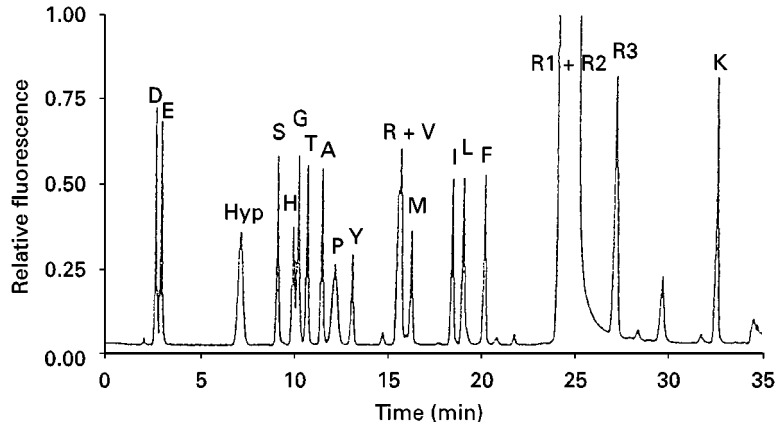

Figure 6 (for details see Table 3) HPLC of AAs derivatized with 9-fluorenylmethyl chloroformate (FMOC). Peaks labelled with one-letter abbreviations for protein AAs, as well as: Hyp, hydroxyproline; R1, FMOC-hydroxylamine; R2, FMOC-hydroxyde; R3, reagent peak present in blank derivatization. (Reproduced with permission from Qu K *et al.* (1996) *Journal of Chromatography* 723: 219.)

Table 4 Advances in the chiral separation of amino acids by LC: applying chiral mobile-phase additives (CMA), chiral stationary-phase columns (CSP) and chiral derivatization reagents (CDR), such as OPA/ NAC[a] and OPA/NIBC[b]

Author and date	*Column size*			*Type*	*Eluents (elution temperature °C): chiral recognition method*	*Detector UV (nm) FEx/Em*	*Reagent (°C)*	*Analyte (pmol L^{-1})*	*RSD %*	*Matrix*	*No. of AAs/elution time (min)*
	cm × mm,		*μm*								
Takeuchi, 1992	15	0.35	5	Develosil ODS-5	A: 40 mmol L^{-1} AmmAc + 27 mmol L^{-1} γ-CD/ ACN = (3:1, v/v) B. AmmAc/ACN = (72:28, v/v); (25°C) CMA	F 315/539	DANS (−)	(−)	(−)	Model study	1 pair/30
Marchelli, 1996	10 15	8 4	5 5	Radialpak C18	Isocratic: ACN/ 30 mmol L^{-1} NaAc (pH 7.0), containing 0.5 mmol L^{-1} N2-*S*-2′-hydroxypropyl-*S*-phenyl-alaninamide + 5 mmol L^{-1} Cu(II) Ac = (2:8, v/v); (21.5°C) CMA	F 330/560	DANS (−)	(−)	(−)	Model study	3 pairs/130
Galli, 1994	15	4 modified	5	LiChrosorb Si 100[c]	Isocratic: ACN/ 10 mmol L^{-1} NaAc (pH 7.52), containing 25 mmol L^{-1} Cu(III) Ac = (7:3, v/v); (60°C) CSP	UV 254	DANS DABS (−)	(−)	(−)	Model study	3 pair/30 4 pair/30
Iida 1997	15	6	5	Home made[d]	A: 100 mmol L^{-1} AmmAc (pH 6.5), B: 100 mmol L^{-1} AmmAc (pH 65)/CH_3OH = 50:50 (v/v); A and B both contain 1 mmol L^{-1} butanesulfonate; (20–30°C) CSP	UV 254	PTC (−)	1000	(−)	Protein sequencing	18 pairs + 1 single/ 150
Nimura, 1986	20	6	5	Develosil ODS-5	A: 50 mmol L^{-1} NaAc B: ACN (25°C) CDR[c]	F 360/405	OPA/ NAC (−)	5000	< 2.3	D- and L-AAs in protein hydrolysates	14 pairs/70
Brückner, 1995	25 + guard 2	4 2.1	5	Hypersil ODS	A: 23 mmol L^{-1} Na acetate (pH 5.95) B: ACN/CH_3OH = (60:5, v/v); (25°C) CDR[d]	F 230/445	OPA/ IBLC (IBDC) (−)	1–1000	< 2	D- and L-AAs in food hydrolysates	17 pairs + 5 single/70

Indications as in Tables, as well as: CD cyclodextrin; [a]*N*-acetyryl-L-cysteine; [b]IBL (D) C, isobutyryl-L(D)-cysteine; [c] [(*S*)- and (*R*)-phenylalanine-amide were covalently bonded to LiChrosorb Si100 silica gel; home made[d] silica support treated with PITC + β-CD; DANS, dansyl; DABS, dabsyl; PTC, phenylthiocarbamyl.

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is experienced; histidine and tyrosine give their mono- and disubstituted derivatives in varying ratios. With longer reaction times, the amount of disubstituted histidine decreases and that of tyrosine increases, together with interfering hydrolysis products of the reagent. At pH ~ 11.4 faster reaction and less interfering hydrolysis products are found. After 40 min reaction time, the monosubtituted histidine and the disubstituted tyrosine are formed in quantitative yield. Also 30% less hydrolysis product is obtained, favouring the resolution of the neighbouring alanine. The separation of the FMOC derivatives and the presence of interfering substances are shown in **Figure 6.**

Chiral Separations (Table 4 and Figure 7)

The knowledge of the distribution of AA enatiomers in different matrices, and/or the enantiomeric purity of AAs, is of primary importance in the quality control of peptide syntheses for pharmaceuticals/ medicines, as well as in various plant products and in high AA-containing foods, including baby formulas.

For separation and quantitation of enantiomers, HPLC is the method of choice. The application of the three main approaches for enantiomer separation is shown in **Table 4** and **Figure 7**, including direct, chiral mobile-phase additive (CMA) and chiral stationary phase (CSP) and indirect methods (chiral derivatization reagent CDR).

Direct Methods

Applying either γ-cyclodextrin (γ-CD) or Cu(II) salts together with N^2-*S*-(N^2-*R*-)2′-hydroxypropyl-*S*-phenylalanineamide as CMA is very time-consuming (**Figure 7**A): the separation of three AA pairs requires more than 2 h. Thus, CMAs can be regarded as an inferior approach in the chiral recognition of AA enantiomers, due to the need for a continuous supply of the often expensive CMA and to the disadvantageous chromatographic conditions. Bonded N^2-*S*-(N^2-*R*-)2′-hydroxypropyl-*S*-phenylalanineamide, CSP, allows the comparison of the CMA and CSP protocols for the same enantiomer separations. The CSP method resolved four pairs of

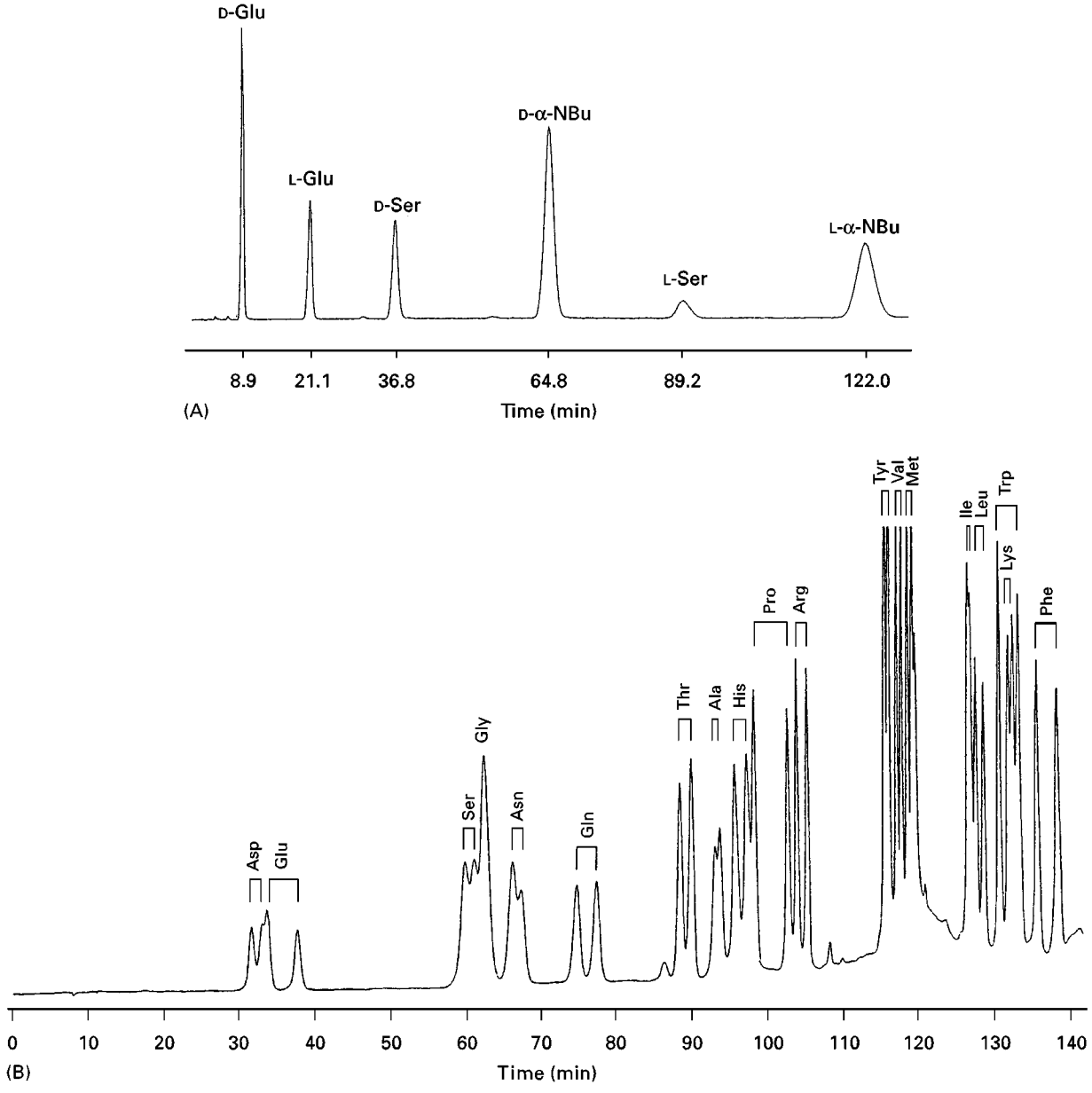

Figure 7 LC separation of AA enantiomers. (A) Enantiomeric separation of a mixture of three dansyl AAs. (Reproduced with permission from Marcelli R *et al.* (1996) *Chirality* 8: 452.) (B) Separation of 37 phenylthiocarbamyl AAs. (Reproduced with permission from Iida T *et al.* (1997) *Analytical Chemistry* 69: 4463.) (C) Aminogram of fir honey derivatized with (a) OPA/IBLC and (b) OPA/IBDC. (Reproduced with permission from Brückner H *et al.* (1995) *Journal of Chromatography* 697: 229.)

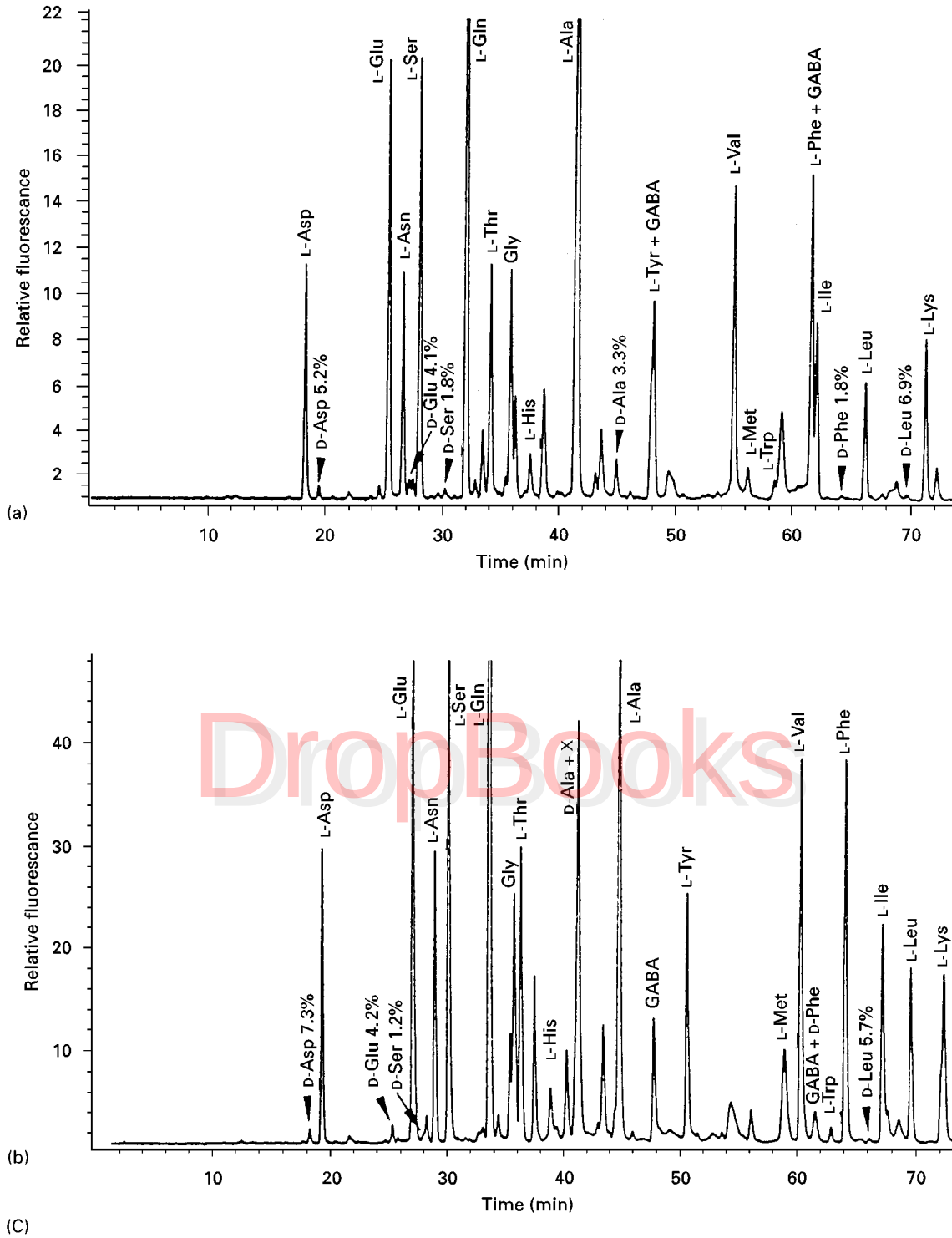

Figure 7 *Continued*

dansylated AAs within 30 min, attesting to the superiority of CSP over CMA. Recently, the elution of the PTC AAs on a new CSP (**Figure 7**B) permitted the partial separation of 18 AA pairs within 150 min.

Indirect Methods

Spectacular results have been achieved with the separation of AAs derivatized by CDRs (**Figure 7**C). Performing the separation with both OPA/*N*-L(D)-

acetyl-cysteinyl and with OPA/*N*-L(D)-isobutyrylcysteinyl AA derivatives gave excellent resolution of enantiomers. Consequently, the CDR technique is the primary importance in a number of practical applications of the separation of enantiomeric AAs. The interaction of AAs with the enantiomerically pure reagents takes place at ambient temperature, without racemization, resulting in the formation of stable diastereomer derivatives.

Online LC-MS

In the case of AAs, thermospray ionization has been displaced by the milder techniques of electrospray (ES) and atmospheric pressure chemical ionization (APCI), converting analyte molecules without fragmentation into ions. The analyte should contain the AAs in a stable form: either in the free condition or in the form of stable derivatives, such as phenylthiohydantoins (PTH) or PTCs. Significantly reduced flow rates are essential (100–300 nL min^{-1}) for stable ES and APCI operation. In automated Edman microsequencing, the ES-MS of PTH derivatives. The protonated molecules were measured with a linear response in the 50–1000 fmol level.

Future Trends

Efforts are needed to extend the life time, plate number and reproducibility of columns, and to standardize testing methods. The extended use of thermostated columns is desirable in order to obtain reproducibility in absolute and relative retention times. LC-MS will be more widely used in laboratories as the cost of these instruments falls to the level of GC-MS, and/or an all-purpose interface becomes available.

See also: **II/Chromatography: Liquid:** Derivatization; Mechanisms: Reversed Phase.

Further Reading

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Thin-Layer (Planar) Chromatography

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Introduction

Thin-layer chromatography (TLC) is a simple and inexpensive technique permitting a number of samples to be handled simultaneously, thus yielding a higher precision than sequential analysis. The inert character of the thin-layer material makes it ideally suitable for use with strong corrosive reagents and one can perform many kinds of chemical reactions on the plate, both from the points of view of detecting and locating the spot and of achieving improved separation. Certain groups of interest can be chemically bonded to the reactive groups of support material, e.g. silanization for reversed-phase studies. Impregnation of the adsorbent with a variety of reagents adds an additional feature for influencing the adsorption characteristics without covalently affecting the inert character of the adsorbent. TLC is also successful in providing direct resolution of enantiomers of a variety of compounds by the proper manipulation of the support material. The analysis of amino